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Meat Flavor. I. Fractionation of Water-Soluble Flavor Precursors of Beef

SUMMARY

Water-soluble extracts of beef contain flavor precursors of compounds responsible for a characteristic broiled steak aroma. Dialysis separated the extract into a nondialyzable high-molecular-weight fraction having a brothy odor during boiling, and a low-molecular-weight fraction having a broiled-steak odor when pyrolyzed. The fraction responsible for the broiled-steak aroma was separated on ion-exchange resins. An aromatic fraction was obtained containing amino acids and peptides. Although sugars have been found in previously described flavor fractions, no sugars were found in this material.

Precursors of meat flavor are among the water-soluble components of raw-meat extracts. Although a number of studies have attempted to elucidate development of the characteristic odors, neither specific precursors nor aroma components have yet been identified completely.

In a series of investigations involving commercial meat extracts (heated concentrates of water extracts) as well as cold-water extracts of beef, Wood and Bender (1957) and Wood (1956, 1961) identified an extensive series of nitrogenous compounds (purines, amino acids, and peptides), carbohydrates (free sugars and sugar phosphates), and organic acids. Wood (1961) postulated that the development of the brown color and flavor in the extracts is due to a Maillard reaction between the amino acids and sugars. In model experiments, browning and a "meat extract" flavor were obtained by heating glucose with a synthetic extract, while browning and a bitter flavor resulted from the interaction of glucose with various amino acids at elevated temperatures. Ribose and ribose-5-phosphate were implicated in the browning reaction in aged or fresh ox-muscle extracts. Certain of the meat flavor and aroma precursors are low-molecular-weight compounds and can be separated by dialysis through cellulose membranes. Hornstein and Crowe (1960) found that the diffusate yielded a fraction similar to one obtained from the whole extract when both were pyrolyzed under vacuum. When free amino acids were separated from the diffusate and heated, unpleasant odors were obtained. They concluded that free amino acids, as such, were not flavor precursors. Recently, Macy *et al.* (1964a,b) determined quantitatively that heating the diffusate of a dialyzed water extract resulted in large losses of amino acids and peptides. A corresponding loss was noted among the carbohydrate components, particularly in the glucose content.

All of these studies were done with water extracts or with diffusates of dialyzed extracts. These extracts contained a large number of components, among which it is difficult, if not impossible, to determine the compounds and reactions actually involved in producing aroma. Batzer *et al.* (1960, 1962), however, fractionated a water extract, carrying it through two dialysis steps, Sephadex gel filtration, and separation by ion-exchange chromatography. They isolated a glycoprotein fraction which, when heated with glucose and inosinic acid, resulted in a meaty aroma. A mixture of glucose, inosine, phosphate, and the amino acid components of the glycoprotein also yielded meaty odors after heating. Those authors (1962) stated that ". . . only certain of the amino acids in the glycoprotein are necessary precursors of meat flavor." The particular amino acids and related compounds involved were not determined.

In the work described herein the fractionation procedure of Batzer *et al.* (1960, 1962) was carried out to obtain more information about the separated fractions. Additional data, varying significantly from published results, have provided further information about the composition of flavor precursors.

MATERIALS AND PROCEDURES

Meat fractionation. Bottom round of beef, purchased in a commercial meat market, was cut into pieces and the fat removed as completely as pos-

sible. The meat was ground; suspended in a volume of cold, distilled water approximately equal to 1.5 times the weight of the meat; and extracted for 18 hr, with continuous stirring, at 4°C. The red-colored extract was clarified by filtration through cheesecloth and then by centrifugation to remove finely suspended particles. The extract was fractionated by the method of Batzer *et al.* (1960, 1962) with some modifications. A cellulose dialysis tube containing 300 ml of distilled water was suspended in the meat extract and maintained with agitation for 18 hr at 4°C. (The contents were then designated extract A.) The slightly colored contents of the dialysis tubing were transferred to Visking No-Jax sausage casing and dialyzed for 18 hr at 4°C against 500 ml of distilled water. The dialysate remaining in the sausage casing is designated *Aa,* and the diffusate as *Ab*. The solutions were freeze-dried. The resultant yellow powders were very hygroscopic, so they were dissolved in small quantities of water and stored at −18°C until used. Fraction *Aa* was separated on a column of Sephadex G-25, the elution being carried out with distilled water. One hundred 3-ml fractions were collected, and the absorbance at 290 mμ was determined with a Bausch and Lomb Spectronic 505 spectrophotometer. Three fractions, designated Aa_1, Aa_2, and Aa_3, were obtained.

Fraction *Ab* was further separated on a column of Dowex 50W [H^+] ×8, 50–100-mesh. Following addition of *Ab* to the column, 150 ml of water was passed through the column followed by 150 ml of 2*M* NH_4OH. The eluates were collected in 3-ml fractions, and absorbance was determined at 290 mμ. Three fractions, designated Ab_1, Ab_x, and Ab_2, were obtained.

Identification of fraction components. *Carbohydrates.* The presence of carbohydrate in the various fractions was determined qualitatively by a simple spot test with 0.5% anthrone in glacial acetic acid (3 drops of sample, 3 of anthrone solution, and 9 of conc. H_2SO_4). Specific identification was made with paper chromatography on Whatman No. 1 paper using *n*-butanol–acetic acid–water (65:15:25) solvent. Silver nitrate and aniline hydrogen phthalate (Block *et al.,* 1952) were used as general sprays for the carbohydrates. A modified Dische spray reagent (0.5% cysteine HCl in conc. H_2SO_4; heat 5–10 min at 85°) was used to identify deoxyribose.

Acids. Organic acids were determined by paper chromatography with the same solvent as above. Bromphenol blue was used as the spray.

Purine compounds. Purines, nucleosides, and nucleotides were separated on paper chromatograms with the butanol–acetic acid–water solvent and identified by their absorption in ultraviolet light at 253 mμ. These compounds also gave blue spots when treated with the silver nitrate-bromphenol blue (SB) reagent of Wood (1955). Further identification of the bases and nucleic acid derivatives was made by their absorption spectra in the ultraviolet (Beaven *et al.,* 1955).

Amino acids. Amino nitrogen was located qualitatively in the various fractions by spotting on Whatman No. 1 paper, spraying with 0.2% ninhydrin in alcohol, and heating for 5 min at 90°. Amino acids were identified by two-dimensional paper chromatography according to the method of Rockland and Underwood (1954) and Underwood and Rockland (1954). Further identification and quantitative data were obtained by analyzing the amino acids before and after hydrolysis for 18 hr in constant-boiling HCl at 110°. The method of Spackman *et al.* (1958) was followed, using a 150-cm column for the neutral and acidic amino acids and a 15-cm column for the basic amino acids. The analyses were done on the automatic analyzer of the Phoenix Precision Instrument Co.

Aroma. Fractionation of precursors of meat aroma was followed by the development of aroma in 0.1-ml samples heated to dryness in small beakers on a hot plate. The temperature at the bottom of the beaker was 150–160°C, as determined with a chromel-alumel thermocouple. Odor identification was made by laboratory personnel, then confirmed by other members of the staff.

RESULTS AND DISCUSSION

Water extraction of ground beef left a grayish-white fibrous residue. When the residue was formed into a hamburger patty and grilled, no browning or other color change occurred, and there was no development of the characteristic meaty aroma and flavor. The hamburger was tough and tasteless. All of the aroma-producing components were in the water extract. On heating of the solution, a profile of odors was noted beginning with a serumy or blood-like odor in the cold extract. As the extract boiled, accompanied by a flocculent precipitation of protein, the aroma formed was described as brothy and buttery, or oleaginous. After complete evaporation of the water, the remaining constituents underwent pyrolysis at higher temperatures. Browning occurred with the development of various odors, culminating in an aroma resembling that of broiling steak.

The scheme for the dialysis and fractionation of the extract is shown in Fig. 1. The first dialysis into the cellulose tubing sepa-

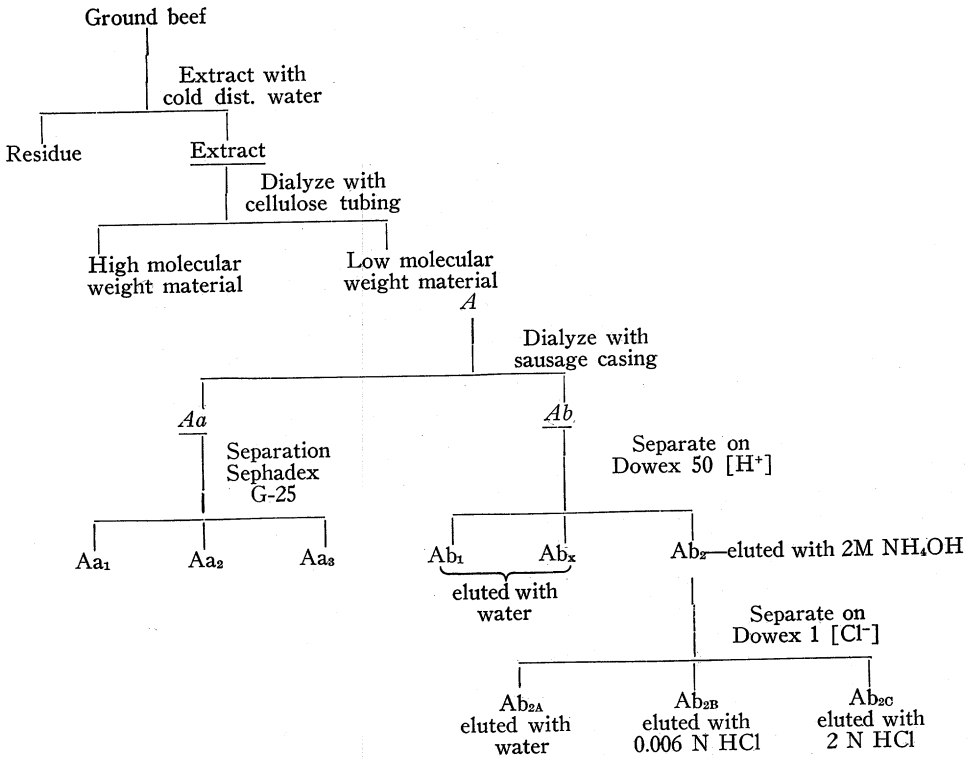

Fig. 1. Fractionation procedure for obtaining a fraction from beef extract containing a meaty aroma.

rated the low-molecular-weight compounds—such as sugars, acids, amino acids, small peptides, and salts—from the proteins. This dialysis step also resulted in separation of the aroma components of the extract. In a thoroughly dialyzed preparation the dialysate retained the substances responsible for the brothy or buttery aroma while the solution was boiling but did not have a characteristic meaty aroma on pyrolysis. The diffusate lacked the full aroma in the boiling stage, achieving at best a mild brothy odor; however, when pyrolysis occurred, a steak-like aroma could be discerned. In all further fractionations using the diffusate fraction of the extract, only the meaty aroma on pyrolysis was observed.

The dialysis of Fraction *A* was carried out in Visking sausage casing. Batzer *et al.* (1960) reported a further fractionation as a result of this procedure. In Table 1, however, the data for *Aa* and *Ab* (columns 1 and 5) indicate that there was essentially an equal distribution of the sack contents across the membrane. It should be noted that the previous authors stated that only particular rolls of Visking sausage casing were able to effect the separation, as a result of small differences in the pore size of the membrane. Reproduction of the reported separation under normal laboratory conditions may be difficult to attain.

The composition of the three fractions obtained by fractionating *Aa* on Sephadex G-25 is shown in Table 1, columns 2, 3, and 4. Since neither Aa_1 nor Aa_3 produced a meaty odor on heating, they were not investigated further; Aa_2 (which did have the characteristic aroma) was freeze-dried. The material was hygroscopic and began to absorb moisture immediately, so the powder was dissolved in a small quantity of water. The solution began to darken, and within a few hours was dark brown. The solution developed an aroma reminiscent of commercial meat extract.

Fraction *Ab* was separated on Dowex 50 [H^+]. Examinations of the fractions

Table 1. Composition of fractions obtained by the separation of meat extract through dialysis and Sephadex or ion-exchange chromatography.

	1 Aa	2 Aa_1	3 Aa_2	4 Aa_3	5 Ab	6 Ab_1	7 Ab_x	8 Ab_2	9 Ab_{2A}	10 Ab_{2B}	11 Ab_{2C}
Meat aroma	+[a]	±	+	−	+	−	−	+	+	+	−
Anthrone test	+	−	+	−	+	+	−	−	−	−	−
Glucose	+		+		+	+	−	−			
Deoxyribose	+		+		+	+	−	−			
Acids	+		+		+	+	−	−	−	−	−
Succinic	+		+		+	+	−	−			
Unknown	+		−		+	−	−	−			
Ninhydrin test	+	+	+	+	+	±	+	+	+	+	−
Alanine[b]	+		+		+	−	−	+	+	+	
Arginine	+		+		+	−	−	+	+	−	
Glutamic	+		+		+	−	−	+	+	−	
Glutamine	+		+		+	−	−	+	−	+	
Glycine	+		+		+	±	+	+	−	−	
Hydroxyproline	+		+		+	−	−	−	−	−	
Leucine (iso)	+		+		+	±		+	+	−	
Methionine	+		+		+	−	−	+	+	−	
Hypoxanthine	+		+		+	−	−	+	−	+	+
Inosine	−		±		±	−	−	±			
Abs. max., mμ	248 400		248 406		248	253	246	248			
Unknown UV absorb. spot	−		−		+	−	−				

[a] + = present; − = absent.
[b] The amino acids were determined by paper chromatography.

at 290 mμ showed three peaks: Ab_1 and Ab_x had been eluted from the resin with water, while fraction Ab_2 came off with $2N$ NH_4OH; Ab_1 contained carbohydrates while fractions Ab_x and Ab_2 were ninhydrin-positive. After freeze-drying, the meaty aroma was found only in pyrolyzed solutions of Ab_2. The composition of the three fractions is shown in Table 1, columns 6, 7, and 8. Glucose, deoxyribose, succinic acid, and traces of leucine and glycine were found in Ab_1, and only glycine in Ab_x. Fraction Ab_2 contained only amino acids, hypoxanthine and a trace of inosine. Large sheets of Whatman No. 1 filter paper (18¼ × 22½ inches) were streaked heavily with a solution of Ab_2 and chromatographed overnight. Strips were cut from the edge of the sheet and sprayed with ninhydrin reagent to locate the amino acids. Another set of strips was observed under ultraviolet light, and absorbing spots marked; the strips were then sprayed with the SB reagent. Amino acid areas were cut out, eluted with water, and rechromatographed for identification after concentration. Two of the spots that reacted with the ninhydrin spray could not be identified. Two areas absorbed in ultraviolet light. The R_f of one spot coincided with a hypoxanthine standard that was run concomitantly on the filter paper. Both the hypoxanthine standard and its corresponding component in Ab_2 reacted positively with the SB reagent, but the second spot absorbing in the ultraviolet light did not react with this reagent. The R_f value of this second spot did not coincide with those of inosine or inosinic acid. The ultraviolet-absorbing areas corresponding to hypoxanthine standard and the two components of Ab_2 were eluted and their ultraviolet absorbance spectra determined. One component of Ab_2 was identified as hypoxanthine, by its spectral characteristics and its behavior on paper chromatography. The second component, although absorbing in uv at 253 mμ, did not show a characteristic spectrum and was not identified.

Hypoxanthine and inosine have been demonstrated in meat extract fractions, and

a requirement for inosinic acid (or inosine plus phosphate) has been reported for the development of meat aroma (Batzer *et al.*, 1962). However, in the fractions obtained in these experiments, only hypoxanthine, with occasional traces of inosine, was found. The hypoxanthine results from the degradation of inosine and inosinic acid, either enzymatically during the storage of the meat or chemically during the procedures to which the extract was subjected. Hypoxanthine itself has not been implicated in flavor development. Fraction Ab_2 consisted of amino acids and the purine; it also had a meaty aroma.

Attempts failed to collect sufficient pure fractions of Ab_2 by paper chromatography to allow the study of aroma development on pyrolysis. Inconclusive odors were obtained with the various components. Ab_2 was then separated on an ion-exchange resin column, Dowex 1 [Cl^-], and eluted with water, 0.006*N* HCl, and then 2*N* HCl. The composition of these fractions is shown in Table 1, columns 9, 10, 11. The water eluate (Ab_{2A}) contained alanine, glutamine, glycine, arginine, methionine, and leucine. The dilute HCl fraction (Ab_{2B}) contained hypoxanthine, alanine, and glutamine. The more concentrated HCl eluate (Ab_{2C}) contained material that absorbed in the ultraviolet light but did not react with the SB reagent. On pyrolysis of these solutions, both the water and dilute acid eluates (Ab_{2A} and Ab_{2B}) had an aroma described as meat-like. Recombining the two fractions did not enhance the odor.

In identifying the amino acid components of the various fractions, shown in Table 1, two-dimensional paper chromatography was used and only qualitative results were obtained. The ion-exchange procedure of Spackman *et al.* (1958) was then used for two purposes: 1) to obtain quantitative data about the amino acid components; and 2) to determine the absence or presence of peptides and proteins by acid hydrolysis of the material. Analyses were made before and after hydrolysis of Fraction Ab_2. The data are shown in Table 2. It was immediately apparent that, instead of the seven amino acids consistently found in this fraction, or the eight amino acids found as a maximum in the meat extract, by paper chromatography, eighteen components were identifiable by the ion-exchange procedure. The discrepancy in the number of amino acids found cannot be explained. Further study with paper chromatography, using different solvents and larger sheets of paper to improve resolution, have not led to the separation of more than eight amino acids by these techniques.

Table 2. The amino acid composition of meat extract fraction Ab_2[a]

	μmoles/mg		
	Unhydrolyzed	Hydrolyzed	Δ
Aspartic acid	.0156	.0933	.0777
Threonine	.0483	.070	.0217
Serine	.186	.105	—.081
Glutamic acid	.082	.357	.275
Proline	.0184	.071	.0526
Glycine	.101	.412	.311
Alanine	.300	.319	.019
Valine	.0695	.104	.0345
Methionine	.0277	.0319	.0042
Isoleucine	.047	.054	.007
Leucine	.092	.107	.015
Tyrosine	.0352	.053	.0178
Phenylalanine	.0375	.048	.0105
Unknown No. 1	....[b]	....	
Lysine	....[b]	.110	....
Unknown No. 2	....[b]	....	
Histidine	....[b]	1.141	....
NH_3	.437	1.38	.943
Arginine	.0388	.0495	.0107
Tryptophan	.0076	....	—.0076

[a] Determined with the automatic amino acid analyzer.
[b] Unknown No. 1 and lysine were unresolved; Unknown No. 2 and histidine were unresolved.

The concentration of lysine and histidine in the unhydrolyzed material could not be calculated, because these peaks were not resolved from the peaks of two unidentified components. Histidine, which appeared to be present in low concentration, was unresolved from a peak which may contain the greatest concentration of amino acid material.

After hydrolysis, significant concentration changes occurred in a number of amino acids. Histidine, while not completely resolved from the unknown material, increased considerably in concentration, whereas its associated unresolved peak decreased in its concentration. Lysine and its unresolved unknown peak separated after hydrolysis; the unknown is still not identified. It was not

possible to determine what changes occurred in the lysine component as a result of the treatment. Aspartic acid, glutamic acid, and glycine increased in concentration after hydrolysis. Serine concentration decreased after hydrolysis. It has been shown that asparagine and glutamine can be eluted with serine (Canfield and Anfinsen, 1963). After acid treatment these dibasic amino acids are converted to aspartic acid and glutamic acid, respectively. This would account for the apparent loss of serine and some of the increase of aspartic and glutamic acids.

The fraction Ab_2 appears to contain peptides or low-molecular-weight water-soluble protein in addition to free amino acids.

The interaction of amino acids with glucose and ribose (or ribose-5-phosphate) by way of the Maillard reaction has been implicated in the development of the meat aroma (Wood, 1961; Hornstein and Crowe, 1964). Batzer *et al.* (1962) isolated a glycoprotein containing glucose that appeared to be part of the aroma-precursor complex. The aromatic fraction Ab_2, described above, however, had no sugar detectable by paper chromatography. The material was further tested for glucose by a specific glucose oxidase procedure (White, 1964) before and after hydrolysis. Fraction Ab_2 was hydrolyzed in 2-ml volumes of 1*N* H_2SO_4, refluxing in a boiling-water bath for 3½ hr. The $SO_4^=$ was precipitated as $BaSO_4$ and the supernatant liquid evaporated to dryness. The hydrolyzed material was redissolved in 2 ml of water for assaying. No glucose was detected in 10-mg samples of Ab_2, either before or after hydrolysis, by this procedure, which is sensitive to 1–5 μg of sugar.

The presence of glucose, and other sugars, was also investigated by the gas-liquid chromatographic method of Sweeley *et al.* (1963). The trimethylsilyl derivatives of the components of Ab_2 were prepared and compared with similar derivatives of glucose, ribose, and deoxyribose. The one small peak present in the Ab_2 GLC chromatogram did not correspond to the peaks for the sugar standards. The peak was not further identified, but since the reagent reacts with other compounds containing −OH groups and amino acids the component is not necessarily a carbohydrate. Thus, on the basis of several types of assays, a fraction of water extract of meat which retains a meat-like aroma does not contain glucose or ribose sugars associated with the Maillard reaction.

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Ms. rec'd 3/18/65.

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